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OVERPRODUCTION OF XYLOSE ISOMERASE AND CONTROL OF *xylA* COPY NUMBER IN LARGE-SCALE FERMENTATIONS OF *ESCHERICHIA COLI*

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ABSTRACT

Cells of *Escherichia coli* have been transformed with the plasmids pTXI-1 and pRK248. Plasmid pTXI-1 contains a copy of the gene which encodes xylose isomerase (*xylA*) and the strong promoter P_L from bacteriophage Lambda. Plasmid pRK248 contains cI857 from Lambda which encodes the temperature-sensitive repressor of promoter function. Upon temperature upshift from 32°C to 42°C cultures of *E. coli* (pTXI-1/pRK248) are induced for massive overproduction of xylose isomerase. The enzyme converts non-fermentable xylose to xylulose - a sugar which can be fermented readily to ethanol. The objectives of this study are to resolve the major problems associated with overproduction of xylose isomerase in *E. coli*. These include, at the molecular level, the reversion and recombination of cI857 and fluctuation in plasmid copy number, the variation in enzyme stability (biochemical level) and, at the physiological level, the need to maintain growth of cultures at high-cell density.

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INTRODUCTION

The heterotrophic eubacterium *Escherichia coli* is the organism of choice for the overproduction of proteins from cloned foreign genes or sequences of DNA harbored in plasmid and bacteriophage vectors. From a commerical perspective, a number of problems may prevent a cost-effective recovery of the desired gene product. These include an inability to achieve optimal and reproducible growths of the host culture, instability of the recombinant plasmid, toxicity of the gene product to the host, and pre- and post-harvest losses in product activity.

In a rich medium the process of synthesizing a cell of *E. coli* from its constituents can be accomplished within 18 min under optimum growth conditions. Important considerations for growth are whether the cultures occur in "closed" or "open" systems, and whether the populations are randomized or synchronized with respect to the growing cells (Dawson 1984). Fed-batch operation of a fermentor is a compromise between ordinary batch and continuous operation (Drew 1981). On a laboratory scale this is achieved by gravity feed or pulse-feeding of primary or secondary substrates. The method permits significant improvements in cell-mass and product productivity and gives the operator the facility to control transient changes between stable growth rates. Of prime concern is the cost of the nutritional supplements and their effects on growth (Cooney, 1982).

Plasmid stability

For gram-negative eubacteria, plasmid replication may be stringent or relaxed. Plasmids with a relaxed control over replication may be present in high copy number within cells (Rodriguez and Tate 1983), although they are subject to spontaneous curing (loss from the cells) if not kept under selective pressure and often retard the growth of the host. Plasmid copy number may vary considerably according to the state of growth of the cells (see Potts 1984; Roberts and Koths 1976), which may contribute to erratic yields of a cloned gene product. The extent to which the physiological state of the host cell contributes to the maintenance of plasmid copy number is not extensively studied, although incubation of cells with chloramphenicol has become standard practice for the amplification of plasmid yield from cells (Maniatis et al. 1982).

Numerous expression vectors, derived from *E. coli* plasmids, are available. These expression vectors incorporate well characterized strong promoters such as *lac* UV5, *trp* and λP_L

(Maniatis et al. 1982). For those which contain the latter, transcriptional activity is regulated by the temperature-sensitive lambda repressor cI857 (Glover 1985). The synthesis of the gene product can thus be controlled precisely through regulation of temperature. There is an upper limit to the size of foreign DNA insert which can be maintained in a plasmid vector. In general, fragments less than 10 kbp are most suitable - larger fragments contribute to the instability of the recombinant plasmid.

The enzyme D-xylose isomerase (xylA product in E. coli; Maleszka et al. 1982) catalyzes the conversion of D-xylose, D-glucose and D-ribose to D-xylulose, D-fructose and D-ribulose respectively. The E. coli xylose isomerase is of minimal use in the food industry principally due to its low temperature optimum but it is ideally suited for xylose isomerisation (Lastick et al. 1986). Xylose, present as hemicellulose in hard woods and agricultural wastes can, therefore, be converted to xylulose and then to ethanol through the use of the enzyme in batch or two-step fermentations with an appropriate ethanol-tolerant yeast capable of xylulose fermentation (Lastick et al. 1986).

Growth of cells

High concentrations of many nutrients are inhibitory to a fermentation process. This inhibition can result from non-specific osmotic effects or highly specific effects involving interaction of a chemical nutrient with a particular enzyme or membrane component (Cooney 1982). In addition, nutrients exert metabolic effects at the level of transcription of necessary genes. Product synthesis can,therefore, be affected markedly, even when growth of the cells is not. A common way to overcome these problems is to use a fed-batch or continuous culture method of operation. These procedures allow one to supply the necessary nutrients at the same rate as the demand for the nutrient, and in the process maintain an optimal chemical environment for growth and/or product formation (Cooney 1982).

Typically, as much as 60% of the cost associated with a particular fermentation process derives from the raw materials used. Complex nitrogen sources are more beneficial to a fermentation than simple ammonia or ammonium salts. Materials such as corn steep liquor, protein hydrolysates and yeast extracts offer greater advantages although they may incorporate a significant proportion of not-utilizable material. Simple nitrogen sources are more desirable although the use of defined nutrients depends to a great extent on whether a process is developed as fermentation cost-intensive rather than recovery cost-intensive.

The purpose of this project is to gain a thorough understanding of the variation in copy number of a xylA plasmid

maintained in cells of E. coli grown to high cell density, to determine the means by which copy number may be maintained, to study reversion and recombination of the cI857 repressor, and to optimize xylose isomerase activity on a cost-effective basis.

PROJECT DESIGN

Growth of E. coli cells to high cell density

The method used to grow cultures to high cell density is based upon that described by Allen and Lull (1986). This method is a glucose gradient-fed fermentation which utilizes an amine-yeast extract-based medium and infusion of oxygen gas. Parameters for efficient growth of cells are being determined in 1.8 l cultures grown in airlift fermentors (BRL/Life Technologies Inc, Gaithersburg, MD and Kontes, NJ). A comparison of the growth characteristics of cells expected from the use of either batch or gradient-fed fermentations is shown in Fig. 1.

Cultures will be fed with different concentrations of glucose (saturation constant K^s for E. coli grown on glucose is 4 to 6.8 x 10^{-2} mg ml^{-1}) to determine cell yield coefficients. Dissolved oxygen, pH and temperature are monitored continuously. A major problem in the current study is to ensure that N levels do not become inhibitory. A range of N sources will be tested (yeast extract, amines, protein hydrolysate).

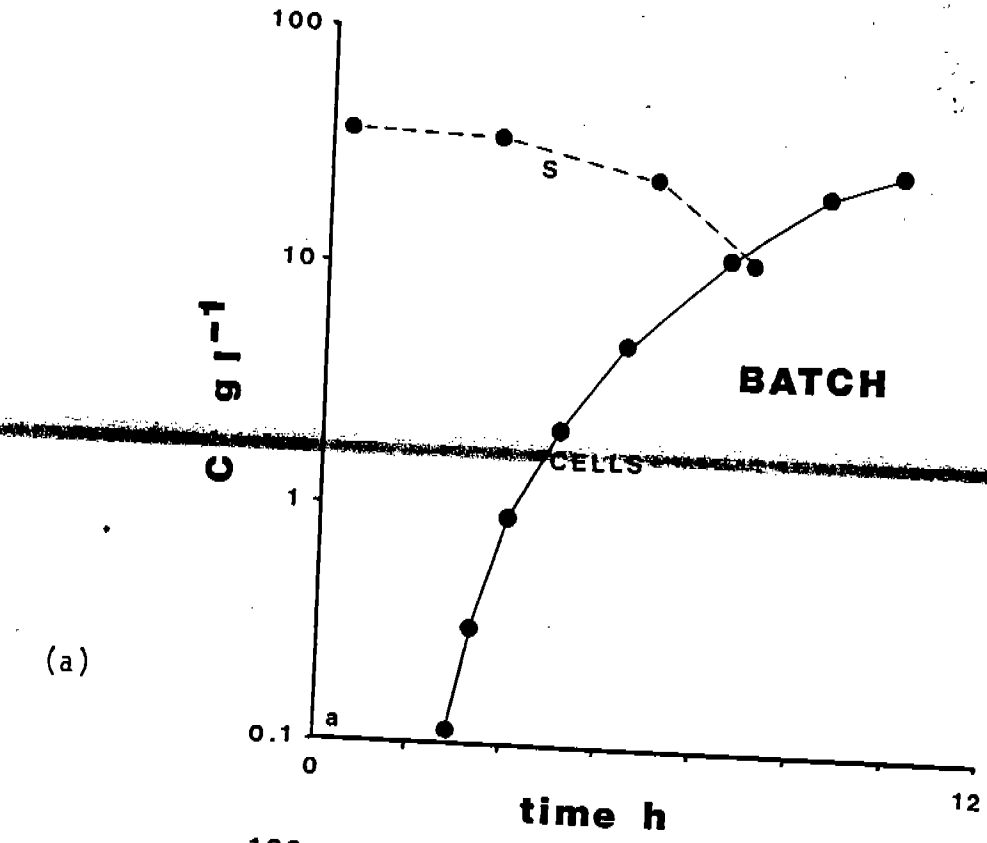
Plasmid copy number

An adaption of the Grunstein-Hogness (1975) technique, as discussed by Shields et al. (1982), is being developed for our system. The technique uses in situ colony lysis with known cell volumes applied to a filter membrane with the aid of a filtration manifold. The amount of specific gene sequence will be determined by hybridization with biotinylated xylA DNA probes. These techniques have been developed in our laboratory for the study of rpoBC expression (Xie et al. 1987).

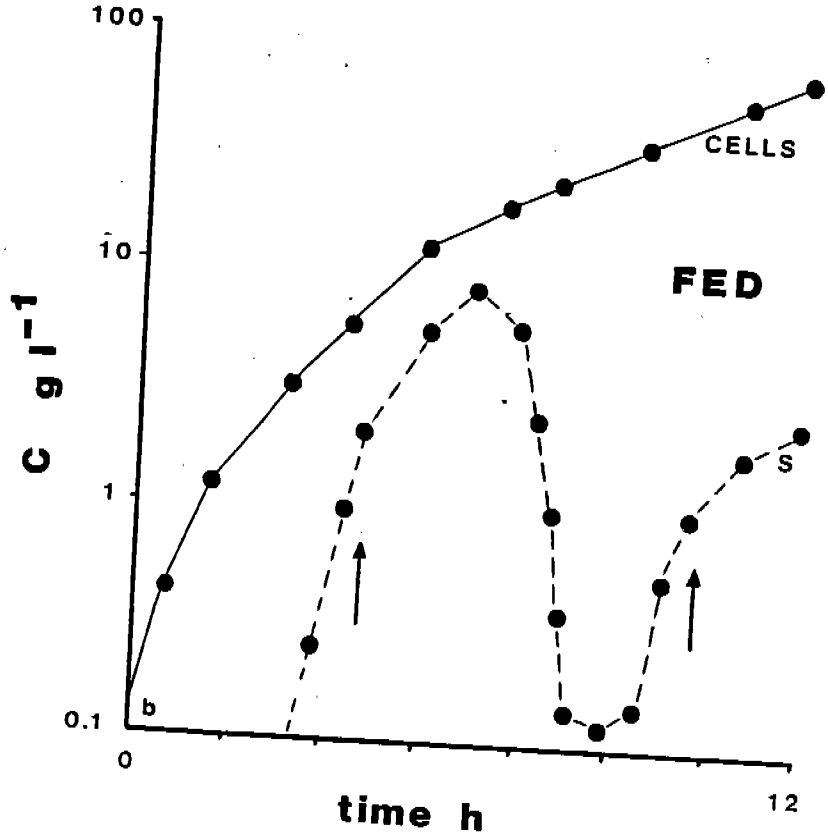
Escherichia coli LE392 (pTXI-1/pRK248) was obtained from Dr S. Lastick at SERI, Golden, Co. A restriction map of pTXI-1 and the orientation of xylA is shown in Fig. 2. Stability of cI857 within pRK248 is of concern as recombination and reversion of cI857 have been noted. A goal of the project is to achieve stability of the repressor to permit the maintenance of control within the system.

Optimization of enyzme activity and stability

Cell aliquots will be removed from reaction vessels at the same time plasmid copy number is determined. Aliquots will be supplemented with ^{35}S-methionine and xylose isomerase production

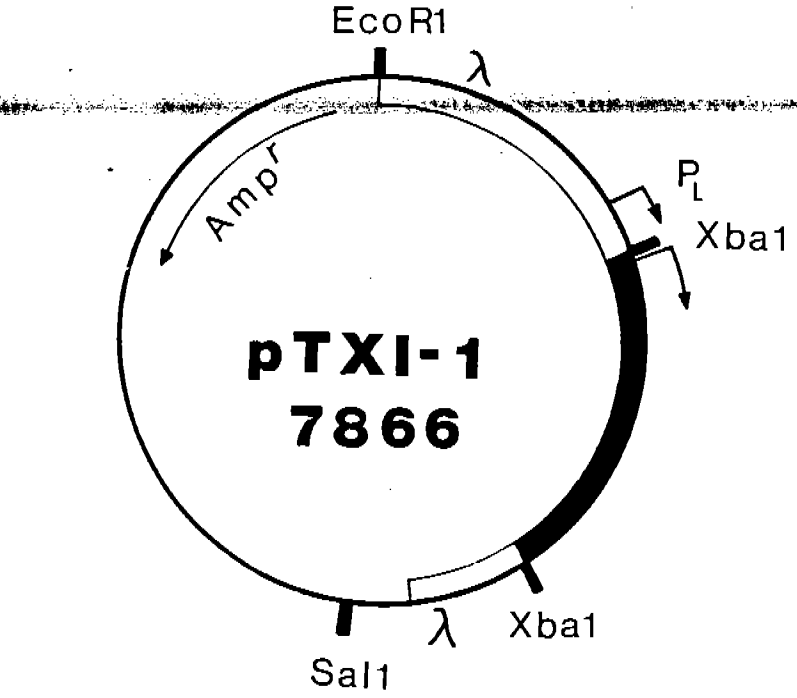


(a)



(b)

Figure 1 Comparison of the growth of Escherichia coli in batch culture (a) and in gradient-fed culture (b). Concentrations (C) of cells and the carbon substrate (S) are indicated in g l^{-1}. In Fig. 1 b arrows indicate times at which the substrate is fed (see Allen and Luli (1986)



xyl A

Figure2 Restriction map of the plasmid pTXI-1(7866 bp; see Lastick et al. 1986).

will be induced through inactivation of the cI857 repressor at 42°C. At time intervals, cells will be lysed and fluorographic analysis with SDS-PAGE will be used to assess enzyme synthesis; simultaneously extracts will be assayed for D-xylose isomerase activity.

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CLONING OF THE XYLULOKINASE GENE FOR IMPROVED YEAST XYLOSE FERMENTATION
I. CLONING THE YEAST XYLULOKINASE GENE BY COMPLEMENTATION OF *E. COLI* XYLULOKINASE MUTATION

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ABSTRACT

Recently it has been implied that yeast xylulokinase (E.C.1.1.9) may play a significant role in the regulation of xylose metabolism in yeasts. A 7.3 kilobase (kb) fragment cloned on YEp13, a yeast–*E. coli* shuttle plasmid, was found capable of complementing *E. coli* xylulokinase mutations. Through restriction analysis and subcloning, the yeast xylulokinase structural gene has been localized on a 2 kb XhoI fragment near the 5' terminal of the cloned fragment and the orientation of the cloned gene has also been determined. Furthermore, the cloned gene most likely is dependent on a plasmid-borne promoter for its expression in *E. coli*.